INHIBITION OF ARYLAMINOPEPTIDASES AND PROLINE IMINOPEPTIDASES OF HUMAN WHOLE SALIVA BY BENZETHONIUM CHLORIDE (BENZYLDIMETHYL [2-[2-(*p*-1,1,3,3-TETRAMETHYLBUTYLPHENOXY)ETHOXY]-ETHYL] AMMONIUM CHLORIDE)

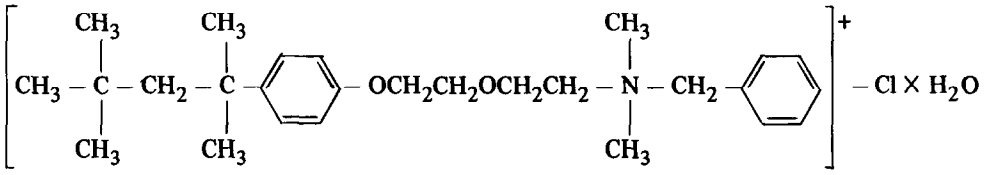
Kauko K. MÄKINEN
Institute of Dentistry, University of Turku, Turku, Finland

1. Introduction

The quaternary ammonium compound benzethonium chloride (benzyldimethyl {2-[2-(*p*-1,1,3,3-tetramethylbutylphenoxy)ethoxy]-ethyl} ammonium chloride) (Mann Research Laboratories, Inc., New York, USA) has been reported among other compounds to inhibit trypsin action [1]. This has been later confirmed in studies in which benzethonium chloride was also found to inhibit the proteolytic activity of rabbit serum [2]. Fibrinolysin was also inhibited but the compound had no inhibiting effect on the action of chymotrypsin, pepsin, or lipase. This paper will provide information about the effect of benzethonium chloride on some aminopeptidase-like enzymes partially purified from human whole saliva. These enzymes, cleaving the 2-naphthylamides of amino or imino acids, have been described more thoroughly elsewhere [3–5]. Benzethonium chloride was found to inhibit selectively the enzymes studied. In cases where inhibition was observed, it was found to be of a basically competitive nature. The enzymic hydrolysis of L-prolyl-2-naphthylamide was inhibited to a lesser extent than that of other amino acid 2-naphthylamides tested.

2. Experimental

Two different salivary proline iminopeptidases, specifically cleaving the 2-naphthylamide of L-proline and L-hydroxyproline were partially purified from human whole saliva as described elsewhere [5]. The enzyme preparations obtained were found to be almost free of enzyme activity against other amino acid 2-naphthylamides. These two proline iminopeptidases are later called I and II. In addition to these two enzymes, an arylaminopeptidase of human whole saliva with a wider specificity and acting most rapidly on the 2-naphthylamides of L-alanine, L-arginine, L-leucine, L-methionine, L-lysine, and L-phenylalanine, was partially purified for this study. In the purification of this arylaminopeptidase the same methods in handling and collecting the whole saliva samples were employed as earlier [5,6]. After these preliminary measurements the crude protein solution was fractionated through DEAE-cellulose columns in exactly the same method used for the proline iminopeptidases [5]. When the fractions were tested with L-leucyl-2-naphthylamide, the arylaminopeptidase peak fractionating last from the column was used in the experiments of



this study. This enzyme preparation differed clearly from the two proline iminopeptidase preparations in possessing only slight activity towards L-prolyl- and L-hydroxyprolyl-2-naphthylamide. Other differences between salivary proline iminopeptidases and aminopeptidases are presented elsewhere [3,4]. The method used in estimating the enzyme activity was based on measuring the amount of liberated 2-naphthylamine by coupling it to diazotized 4-amino-1,3'-dimethylazobenzene as described in more detail elsewhere [5]. Substrate concentrations were varied 400-fold, from 1.66×10^{-4} M to 6.66×10^{-2} M, and benzethonium chloride concentrations 100-fold, from 1.66×10^{-6} M to 1.66×10^{-4} M. For practical reasons, the data of only a few substrate concentrations are given.

3. Results and discussion

The kinetic data obtained when studying the effect of benzethonium chloride on the action of the amino- and iminopeptidases involved was plotted mainly in the following ways: $1/v_i$ against [I], $1/v_i$ against $1/[S]$, v_0/v_i against [I], and v_i against [S], where v_i is the rate in the presence of the inhibitor, and v_0 that in its absence. [I] is inhibitor concentration and [S] is that of the substrate. The results obtained can be briefly summarized as follows.

The enzymic hydrolysis of L-prolyl-2-naphthylamide was inhibited hardly at all, or only to a slight extent, by benzethonium chloride. On the other hand, the hydrolysis of all other amino acid 2-naphthylamides tested (that of L-leucine, L-alanine, and L-lysine) was markedly inhibited by benzethonium chloride. The exact expression of the inhibition by benzethonium chloride was rendered difficult due to the simultaneously occurring inhibition by high concentrations of substrate. In cases where inhibition was found, no differences were observed in the pattern of the inhibition by benzethonium chloride when different enzymes were used. On the other hand, the degree of substrate inhibition varied with different substrates. All of the plotting methods used corroborated the results obtained by each other.

The types of inhibition revealed are illustrated as some examples. Fig. 1 shows a plot of $1/v_i$ against [I] with L-prolyl-2-naphthylamide; in this type of plot, noncompetitive inhibition is indicated if the lines at different substrate concentrations meet at a point on the abscissa. The results of fig. 1 suggest that the hydrolysis of L-prolyl-2-naphthylamide by proline iminopeptidase I was slightly and most probably competitively inhibited by benzethonium chloride. K_i values between 2.0×10^{-4} M and 2.5×10^{-4} M were obtained in this case. At very high substrate concentrations the pattern of inhibition was changed, pro-

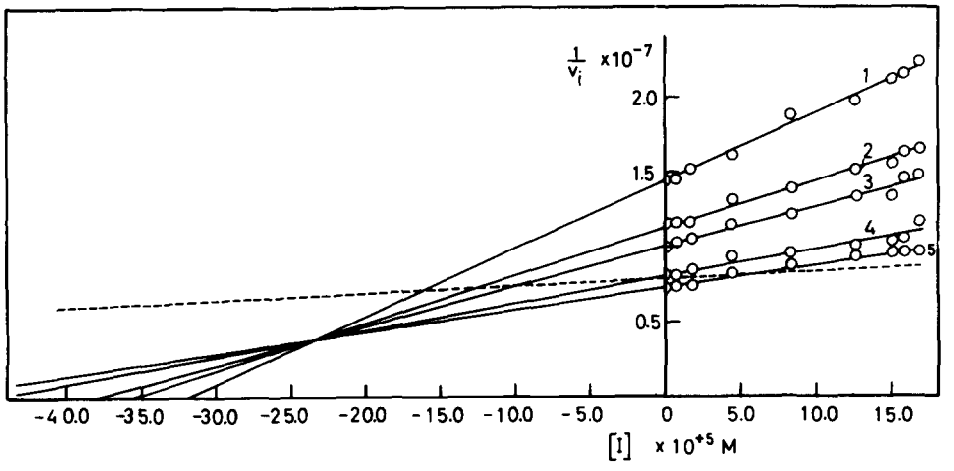


Fig. 1. Plot of $1/v_i$ against [I] for the proline iminopeptidase I-catalyzed hydrolysis of L-prolyl-2-naphthylamide inhibited by benzethonium chloride. Legend: 1, $[S] = 1.66 \times 10^{-5}$ M; 2, $[S] = 3.33 \times 10^{-5}$ M; 3, $[S] = 5.00 \times 10^{-5}$ M; 4, $[S] = 8.35 \times 10^{-5}$ M; 5, $[S] = 1.66 \times 10^{-4}$ M and 2.50×10^{-4} M. The figure also shows a curve drawn (dotted line) using the data obtained with proline iminopeptidase II (substrate concentration: 0.83×10^{-3} M).

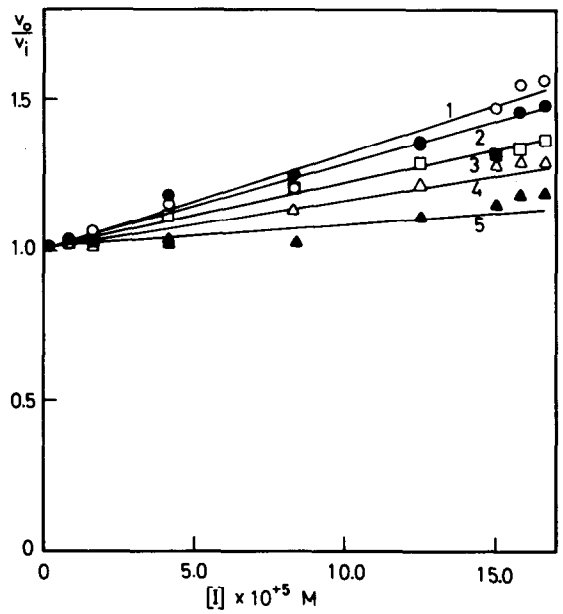


Fig. 2. Plot of v_0/v_i against [I] for the proline iminopeptidase I-catalyzed hydrolysis of L-prolyl-2-naphthylamide inhibited by benzethonium chloride. Legend: 1, [S] = 5.00 X 10^{-5} M; 2, [S] = 3.33 X 10^{-5} M; 3, [S] = 1.66 X 10^{-4} M; 4, [S] = 2.50 X 10^{-4} M; 5, [S] = 3.33 X 10^{-3} M.

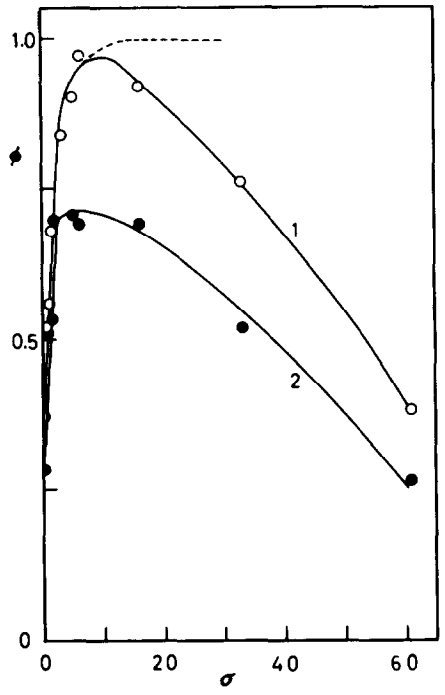


Fig. 3. Plot of relative velocity, ϕ, against relative substrate concentration, σ, for the arylaminopeptidase-catalyzed hydrolysis of L-leucyl-2-naphthylamide inhibited by high concentrations of substrate. Legend: 1, without added benzethonium chloride; 2, with 1.66 X 10^{-5} M benzethonium chloride. The dotted curve illustrates the curve without excess substrate inhibition.

ducing a curvature less easy to explain. It is also possible that the plots obtained in this study represent mixed inhibition. There was a slight curvature indicating that the dependence on the inhibitor concentration was to a power greater than unity. The K_i values given may be termed apparent ones. The same figure also shows one example from results obtained with proline iminopeptidase II. This enzyme was affected hardly at all by benzethonium chloride at any substrate concentration tested.

Fig. 2 shows a plot of v_0/v_i against [I] for similar data. The results of this figure confirm the assumption made about the likely competitive inhibition. When plots of $1/v_i$ against 1/[S] were made, substrate inhibition observed with proline iminopeptidase I was seen to be strong, and that with proline iminopeptidase II slight. In the former case plotting of $1/v_i$ against [S] gave for K's (the dissociation constant for enzyme substrate complex which is ineffective), a value of 4.0 X 10^{-3} M, determined as Dixon and Webb [7] have suggested. In the arylaminopeptidase-catalyzed hydrolysis of L-leucyl-2-naphthylamide inhibition by high concentrations of substrate was observed, although this inhibition was less than with prolyl-2-naphthylamide mentioned above. However, all plots clearly revealed this phenomenon. One demonstrative way to describe substrate inhibition is shown in fig. 3, where ϕ is plotted against σ where $\phi = v_0/V$ and $\sigma = [S]/K_m$ [7]. In these expressions V is the maximum velocity and K_m is the Michaelis constant. When the apparent values of 1.0 X 10^{-7} M min^{-1} and 1.0 X 10^{-4} M, determined graphically for V and K_m, respectively, were used to calculate the relative substrate concentration σ and the relative velocity ϕ, the curve shown in fig. 3 was obtained; i.e., an almost typical example of inhibition by high

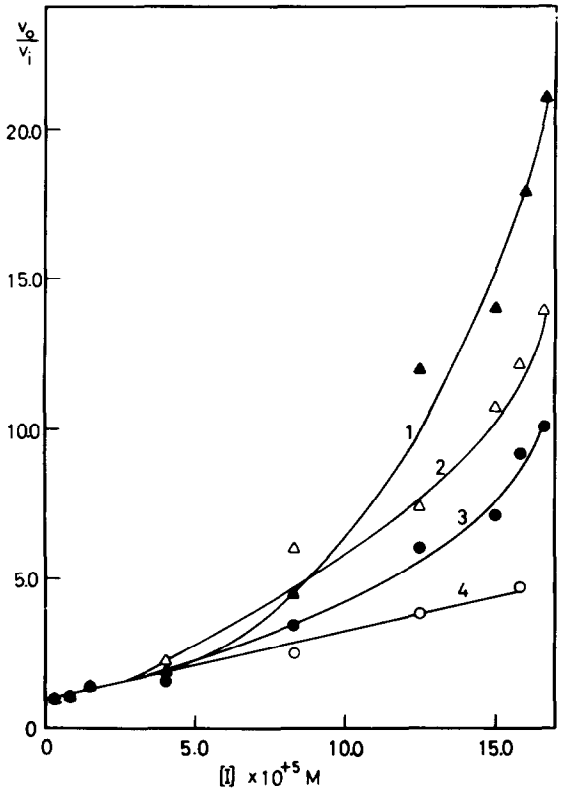


Fig. 4. Plot of v_o/v_i against [I] for the arylaminopeptidase-catalyzed hydrolysis of L-leucyl-2-naphthylamide inhibited by benzethonium chloride. Legend: 1, [S] = 8.35×10^{-5} M; 2, [S] = 1.66×10^{-3} M; 3, [S] = 3.33×10^{-5} M; 4, [S] = 3.33×10^{-3} M.

substrate concentrations. With the 2-naphthylamides of L-alanine and L-lysine no marked substrate inhibition was observed.

With the 2-naphthylamides of L-leucine, L-alanine, and L-lysine, the hydrolysis by the arylaminopeptidase was strongly inhibited in the presence of benzethonium chloride. The apparent K_i values obtained with plots of $1/v_i$ against [I] at low substrate concentrations were approximately 1.5×10^{-5} M, 1.5×10^{-5} M, and 1.6×10^{-5} M for the three amides, respectively.

Fig. 4 shows a plot of v_o/v_i against [I] as an example of the pattern of inhibition observed in the arylaminopeptidase-catalyzed hydrolysis of L-leucyl-2-naphthylamide. Competitive inhibition was the most likely explanation for the results obtained. The curves of the $1/v_i$ versus [I] plot showed pronounced curvature which shows that the dependence on the inhibitor concentration was even in this case to a power greater than unity. Therefore, competitive inhibition in which two (or perhaps more) inhibitor molecules are bound to the enzyme is also a likely possibility.

The results showed that benzethonium chloride could be selectively used as an inhibitor for arylamino- and aryliminopeptidase-like enzymes. The compound is highly water-soluble and produces solutions which can be rather easily buffered even at higher concentrations of the compound. The results also suggest that benzethonium chloride could be employed in controlling the activity of oral peptidases, in particular those derived from oral micro-organisms, known to be a factor in the development of the caries process and other oral diseases.

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